# Analysis of Avermectin Residues in Game Meats (Bison, Deer, Elk, and Rabbit) by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) (CARTS No. IR01040 LIB 2 of 3)

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## Abstract

Avermectins and milbemycins are used as veterinary therapeutic agents to treat and prevent nematode and arthropod parasites in animals[1]. Until recently, the FDA did not routinely test for avermectins in domestic game meats such as bison, deer, elk, and rabbit. This LIB describes a method for the quantitative analysis of 7 avermectins including abamectin, doramectin, emamectin, eprinomectin, ivermectin, moxidectin, and selamectin in bison, deer, elk, and rabbit tissue based on previously published LIB 4496 (FY11)[2], LIB 4552 (FY13)[3], LIB 4567 (FY14)[4] and LIB 4636 (FY18)[5]. Avermectins were extracted from the different matrices with an acidified acetonitrile solution and defatted using an alumina clean-up column followed by LC-MS/MS analysis. Analytes were separated using an Agilent Eclipse Plus C8 liquid chromatographic column and detected using positive mode electrospray ionization (ESI) on a Sciex QTrap 5500 hybrid linear ion trap mass spectrometer. Multiple reaction monitoring (MRM) was performed, fragmenting the [MH]+ or $[MNH_4]$+ precursor ions into their respective product ions. Ammonium adduct $[MNH_4]$+ precursor ions were monitored for ABA, DOR, IVR, while [MH]+ precursor ions were monitored for EMA, EPR, MOX, and SEL. Within the European Union legislation B2a group, ivermectin is the only approved animal drug for bison and reindeer with a tolerance of 15 ng/g in bison liver per 21 CFR 556.344[6]. The method was validated for bison liver and muscle, deer, elk, and rabbit muscle per FDA OFVM Level Two validation[7] criteria. Recoveries were calculated using extracted calibration curves for each type of matrix. The method was validated at the 0.5VL, 1.0VL, and 2.0VL levels where the 1.0VL corresponds to 10 ng/g for all compounds. The average accuracy of fortified compounds in all the matrices ranged from 77.2% to 113% at the target quantitative validation level. Since bison liver can be difficult to obtain, bovine liver was also validated and the recoveries were comparable to the bison liver validation results. Since the initial start of this program in 2014, approximately 100 bison liver samples have been analyzed for avermectins with two samples determined to be positive for ivermectin and eprinomectin at concentrations of 6.97ng/g and 9.32 ng/g, respectively.

## INTRODUCTION

Avermectins and milbemycins are macrocyclic lactone (MLs) drugs widely used in animal husbandry against nematode and arthropod parasites. Abamectin (ABA), doramectin (DOR), emamectin (EMA), eprinomectin (EPR), ivermectin (IVM), and selamectin (SEL) belong to the avermectin group, whereas moxidectin (MOX) is one of the milbemycins[1]. The European Union (EU) conducted an audit in 2010 of the United States Food and Drug Administration programs designed to monitor pesticide residues, chemotherapeutic agents, industrial contaminants, and toxic elements in domestically produced animal derived foods. The outcome of the audit identified differences between the EU and FDA in the design of their respective programs to monitor veterinary residues. In order to address the differences, the Office of Food Safety in the Center for Food Safety and Applied Nutrition (CFSAN) requested that the Office of Regulatory Affairs (ORA) sample animal derived foods for the presence of residues identified by the EU, including ivermectin in domestically raised bison liver.

In response to the EU audit, a method was validated to include seven macrocyclic lactone drugs, not just ivermectin. Discussion between CFSAN, ADRC, and the Denver Laboratory (DENL) resulted in a list of analytes to include in the validation. The USDA Agricultural Research Service (ARS) published a method to qualitatively identify more than 100 veterinary drug residues in bovine muscle[8] and the avermectins were included as part of the analytical methodology. The method required a novel post-column infusion for the avermectin compounds, but the 1X level for ivermectin was 100 ng/g, which is well above the tolerance level of 15 ng/g for bison liver. Methodology for these types of compounds has been previously developed and validated in the Denver Laboratory for aquaculture and milk: LIB 4496 (FY11)[2], LIB 4552 (FY13)[3], LIB 4567 (FY14)[4] and LIB 4636 (FY18)[5].

In this current validation, avermectin and moxidectin was applied to game meats. The method matrix expansion to game meats was validated according to the requirements of an FDA OFVM (Office of Foods and Veterinary Medicine) Level Two Chemical Method Validation[7] with validation levels (VL) at 0.5VL, 1.0VL, and 2.0VL where VL was equal to 10 ng/g for all compounds. Even though the tolerance level for ivermectin in bison liver is 15 ng/g, all avermectin aquaculture methods 1.0VL level at the 10 ng/g. Hence for ease in DENL, 10 ng/g was used for the 1.0 VL level. This validated method is intended for ORA regulatory analysis to test game meats for avermectin and milbemycin residues, and to expand the scope of ORA veterinary drug residue monitoring.

## METHODS AND MATERIALS

### *Equipment*

a) LC-MS instrument. – 5500 Q TRAP hybrid quadrupole linear ion trap mass spectrometer (Sciex, Foster City, CA, USA) utilizing a TurboV™ ion source with the TurboIonSpray® (i.e., electrospray ionization) probe installed and coupled to an Agilent 1200 Series binary pump, degasser, thermostated column compartment (Agilent Technologies, Santa Clara, CA, USA) and HTC PAL autosampler (CTC Analytics, LEAP Technologies, Carrboro, NC, USA). Analyst 1.6.2 software was used to acquire and analyze the data (Sciex).
b) Eclipse Plus-C8 4.6 x 50 mm, 1.8 µm column, (959941-906, Agilent Technologies, Santa Clara, CA, USA).
c) Solid phase extraction (SPE) cartridge – Alumina (P/N 714-0500-E; 25 mL column with 5g, Isolute AL-N, Biotage, Charlotte, NC, USA).
d) Centrifuge – refrigerated to 5 ºC, capable of accelerating 50 mL tubes to 6000 rpm (4032 x g) or equivalent.
e) Vortex mixer – Vortex Genie 2 (Scientific Industries, Bohemia, NY), or equivalent.
f) Sonicating bath – 8892 Ultrasonic Cleaner (Cole-Palmer, Vernon Hills, IL, USA), or equivalent.
g) Evaporator – TurboVap® LV nitrogen evaporator with thermostated water bath set to 50 ºC (P/N 103198, Zymark, Hopkinton, MA), or equivalent.

h) Shaker – 2000 Geno/Grinder (Spex Sample Prep, Metuchen, NJ, USA) or equivalent.
i) Food processor – RobotCoupe Blixer, homogenizer, 4 quart, model RS1BX4V (RobotCoupe USA, Inc., Ridgeland, MS) or equivalent.
j) Centrifuge tubes – 50 mL disposable, conical, graduated, polypropylene tubes with cap (Falcon® Blue Max™, 50 mL tubes P/N 352070, Becton Dickinson, Franklin Lakes, NJ). 1 mL disposable, micro-centrifuge tubes (VWR International Inc., West Chester, PA, USA, P/N 87003-296), or equivalent.
k) Syringe filters – Acrodisc® CR 13 mm syringe filter with 0.2 µm polytetrafluoroethylene (PTFE) membrane (P/N 4542, Pall Life Sciences) with 1-mL disposable syringe (P/N 309602, Becton Dickinson, Franklin Lakes, NJ).
l) Volumetric glassware and pipettors – 100.0 and 10.0 mL volumetric flasks, class A; adjustable volume pipettors with disposable polypropylene tips – 10-100 µL (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY), 200-1000 µL (Ulster Scientific, Inc., New Paltz, NY), and 1-5 mL (Wheaton Science Products, Millville, NJ), or equivalent.
m) Glassware and LC vials – disposable glass culture tubes (20 x 150 mm), disposable Pasteur pipettes; 2 mL glass LC vials with snap caps.

***Reagents and Standards***

a) Solvents. –
    a. Acetonitrile –LC/MS Optima Grade (Fisher Scientific, Pittsburgh, PA, USA).
    b. Water – LC/MS Optima Grade (Fisher Scientific).
    c. Methanol – LC/MS Optima Grade (Fisher Scientific).
b) Reagents-
    a. Sodium chloride (NaCl) – Fisher Bioreagents (Fisher Scientific).
    b. Ammonium formate – Acros Organics (Fisher Scientific, Pittsburgh).
    c. Acetic acid (glacial) – (EMD Chemicals, Inc., Gibbstown, NJ).
c) Extraction solution – 0.10% glacial acetic acid in acetonitrile. 1.00 mL glacial acetic acid diluted to 1000 mL with acetonitrile.
d) LC systems mobile phases –
    a. Mobile Phase A – 10 mM ammonium formate in water – 0.670 g ammonium formate dissolved in 1000 mL de-ionized water. Approximate pH = 6.
    *(Note: Store Mobile Phase a in an amber bottle and protect from light)*
    b. Mobile Phase B – 100 % acetonitrile.
e) Analytical standards. –
    a. Neat Materials – All analytical standards were ordered from Sigma-Aldrich, specifically as Fluka products.
    b. SPEX CertiPrep – Alternatively, custom prepared solutions may be purchased from manufacturers such as SPEX Certiprep. Premix standards are available containing abamectin, doramectin, emamectin, eprinomectin, ivermectin, and moxidectin at 100 µg/mL.
    c. Selamectin was prepared from a neat solution as it was not cost effective to order the standard from SPEX.
b) Negative control – All products (bison and bovine liver and muscle, venison, elk, and rabbit) were acquired from a local market or previous samples tested to determine that specified avermectins were not present above the stated method detection level (MDL).

*Note: Equipment and reagent sources have been provided for information and guidance. Equivalent products may be substituted as appropriate.*

***Standard Preparation***

**a) Neat Materials**

a. Stock Standard Solutions (Continuous Calibration Verification CCV):
Prepare individual stock standards at ~500 μg/mL in methanol, taking into account the content of the active substances (i.e., counter ions and purity) of the avermectins.
*Note: If there is not enough neat material available, concentration maybe varied.*

**Table 1: Example of Stock Standards Solution Prepared from Neat Materials**

| Stock Standards | salt form (g/mol) | Non-salt (g/mol) | Wt. factor | Purity Factor | corr. factor | Actual wt. (mg) | Final Vol. (mL) | Conc µg/mL |
|---|---|---|---|---|---|---|---|---|
| Abamectin (ABA) | 873.1 | 873.1 | 1.000 | 0.969 | 0.9690 | 4.434 | 10.0 | 429.7 |
| Doramectin (DOR) | 899.1 | 899.1 | 1.000 | 0.971 | 0.9710 | 6.194 | 10.0 | 601.4 |
| Emamectin Benzoate (EMA) | 1008.2 | 886.1 | 0.879 | 0.993 | 0.873 | 4.386 | 10.0 | 382.9 |
| Eprinomectin (EPR) | 914.1 | 914.1 | 1.00 | 0.926 | 0.926 | 6.090 | 10.0 | 563.9 |
| Moxidectin (MOX) | 875.1 | 875.1 | 1.000 | 0.959 | 0.959 | 5.604 | 10.0 | 534.4 |
| Ivermectin (IVR) | 875.1 | 875.1 | 1.000 | 0.960 | 0.960 | 4.525 | 10.0 | 434.4 |
| Selamectin (SEL) | 770.1 | 770.1 | 1.000 | 0.990 | 0.990 | 1.586 | 5.00 | 314.0 |

b. Stock Standard Solutions (ICV): A second set of stock solutions is prepared as initial calibration verification (ICV) solutions. These solutions were prepared in the same manner as the stock standard CCV.

c. Working Mixed Intermediate Solution (CCV - Standards & Spiking): Prepare one solution containing 1000 ng/mL of all compounds. This was done by adding the compounds to a 25.0 mL volumetric flask and diluting to the mark with methanol.

**Table 2: Example of Working Mix Intermediate Solution – Standards and Spiking**

| Working Mix Intermediate Solution | Stock Conc. (µg/mL) | mLs Added | Final Volume mL | Final conc. (ng/mL) |
|---|---|---|---|---|
| Abamectin (ABA) | 429.7 | 0.0582 | 25 | 1000 |
| Doramectin (DOR) | 601.4 | 0.0416 | | |
| Emamectin (EMA) | 382.9 | 0.0653 | | |
| Eprinomectin (EPR) | 563.9 | 0.0443 | | |
| Moxidectin (MOX) | 537.4 | 0.0465 | | |
| Ivermectin (IVR) | 434.4 | 0.0576 | | |
| Selamectin (SEL) | 314.0 | 0.0796 | | |

d. Working Mixed Intermediate Solution (ICV- Standards & Spiking): A second Mixed Intermediate Solution was prepared from the ICV Stock Standard Solutions. This solution was prepared in the same manner as the working Mixed Intermediate CCV.

***Note:*** All stock standards solutions were transferred to 20 mL glass scintillation vials and stored at 4 °C. The CCV, ICV, and stock and mixed standard solutions are stable for 1 year[2].

**b) SPEX CertiPrep Custom Mixture**

a. Avermectin mix (100 µg/mL each component), but does not contain selamectin.
b. Prepare the working mixed intermediate solution to 1000 ng/mL in methanol by adding 250 µL of avermectin mix standard (100 µg/mL) and the appropriate amount of selamectin into a 25.0-mL volumetric flask. Diluted to volume with methanol.
c. Prepare the ICV working mixed solution at 1000 ng/mL as stated above, substituting a second set of solutions from a different lot or a different ampule of the same lot.

**IMPORTANT:** Sonicate all SPEX ampules for 15 minutes prior to taking an aliquot for dilution.

***Extracted Matrix Calibrants and Recovery Control Checks for Regulatory Analysis***

Extracted calibration standards were prepared by spiking 3.00 (± 0.050) grams of the appropriate negative control tissue and taking the fortified tissue through the extraction procedure. The fortified extracted calibration standards were prepared at concentrations of 5.00, 10.0, 20.0, 40.0, and 80.0 by adding 15, 30, 60, 100, 120, and 240 µL of the 1000 ng/mL mixed intermediate standard to 3.00 g of homogenized muscle tissue, respectively. For validation, a 100 ng/mL mixed solution was prepared and used to prepare a low concentration extracted standard at 2.50 ng/g. For routine regulatory analysis, this extracted standard was not prepared.

**Table 4: Example of Extracted Calibration Curve Preparation**

| Extracted Curve | Amt added of 1000 ng/mL Working Standard (µL) | Sample weight (grams) | Conc. (ng/g) |
|---|---|---|---|
| Reagent Blank | n/a | 3.00 | |
| Extracted Std-1 | 15 | 3.00 | 5 |
| Extracted Std-2 | 30 | 3.00 | 10 |
| Extracted Std-3 | 60 | 3.00 | 20 |
| Extracted Std-4 | 120 | 3.00 | 40 |
| Extracted Std-5 | 240 | 3.00 | 80 |
| Extracted ICV | 30 | 3.00 | 10 |

***Recovery Control Checks for Regulatory Analysis***

Fortify spike/duplicate by adding 30 µL of working mix solution (1,000 ng/mL) to 3.00 ± 0.05 grams of negative control tissue to yield the 10 ng/g concentration level for all compounds.

Example spiking calculation:

| | | |
|---|---|---|
| 30 µL | 1000 ng | = 10 ng |
| 3.00 grams of negative control | mL | g |

***Sample Homogenization***

Bison, bovine, deer, elk, and rabbit muscle tissue and bovine/bison liver was acquired from a local market and tested before use as a negative control. Approximately 1-2 cups of dry ice were added to pieces of tissue in a food processor and homogenized for ~30 s, producing a dry ice/tissue powder matrix. The dry ice/tissue matrix was transferred to sterile whirl-pak bags. The carbon dioxide was allowed to evaporate in a freezer overnight before tightly sealing the sample for storage at -20 ºC until analysis.

***Extraction Procedure***

This extraction procedure has been reported in LIB 4496[2] and LIB 4567[4]. Three (3) grams (±0.05 g) of ground tissue was weighed directly into a 50-mL polypropylene centrifuge tube and allowed to thaw; fortified calibrant and spike samples were equilibrated for 15 minutes after spiking. To each sample, 0.20 grams NaCl and 10 mL of 0.1% acetic acid in acetonitrile (v/v) was added. The

sample was capped, vortexed for 10 seconds and mechanically shaken for 5 minutes. Samples were placed in a sonicating bath for 5 minutes and then centrifuged at 6000 rpm for 5 minutes at 4ºC. Alumina-N SPE cartridges were conditioned with 4.00 mL of acetonitrile without applying a vacuum or pushing air through the cartridge; however, cartridges were not allowed to go dry. The 10-mL sample extract was loaded onto the conditioned SPE and collected into glass culture tubes, again, only by gravity elution. Cartridges were washed into the collection tubes by gravity with 4.00 mL of acetonitrile added and collected followed by the addition and collection of an additional 2.00 mL of acetonitrile. Sample eluates were evaporated to dryness at 50 ºC with initial 10 psi $N_2$ flow then increased to 15 psi for approximately 30-60 minutes until just dry. The samples were reconstituted with 3.00 mL of diluent, vortexed for 20 seconds, sonicated for 5.00 minutes, and allowed to sit at room temperature for 15 minutes. All samples were transferred to 2 mL capped polypropylene conical vials and centrifuged at 14,000 rpm for 5 min or filtered thru a 0.20 µm PTFE filter. The supernatant was then transferred to HPLC vials for analysis.

***Instrumentation***

*a)* ***LC-MS/MS system*** – The 5500 Q TRAP (hybrid quadrupole linear ion trap (QqLIT) is a combination system in which the final quadrupole can operate as conventional mass filter or as linear ion trap with axial ion ejection. For the purpose of this method the instrument was operated in triple quadrupole mode and calibrated per the manufacturer's instructions. The analyses were performed using electrospray ionization in positive mode. The instrument conditions were as follows: ion spray voltage, 5000 V; curtain gas, 20 (arbitrary units); GS1 and GS2, 40 and 60, respectively; probe temperature, 200 ºC. The entrance potential (EP) was 10 and the dwell time was 50 msec. Nitrogen served as sheath gas and collision gas with a CAD gas setting of medium. MRM experiments allowed the maximum sensitivity to be obtained for the detection of the target molecules. The optimization of MS parameters (declustering potential (DP), collision cell entrance potential (CEP) for precursor ions and collision energy (CE), collision cell exit potential (CXP) for product ions was performed by compound optimization. Table 4 shows the values of the parameters optimized and the MRM transitions used for the confirmation and quantification of avermectin residues.

*b)* ***HPLC system*** – Agilent 1260 HPLC system equipped with pump, solvent degasser, autosampler, and column oven. An Eclipse Plus-C8 4.6 x 50 mm, 1.8 µm column was used and kept at 30ºC oven temperature. The pump was operated at a flow rate of 0.6 mL/min. A binary gradient system was used to separate analytes comprising mobile phase A, 0.1% formic acid in water, and mobile phase B, 0.1% formic acid in acetonitrile. The gradient profile was: (1) 0-4.0min, 60.0%A; (2) 4.00-6.50 min, 0.00%A; (3) 7.00-10.0 min, 60% A; The Agilent autosampler injection volume was 10 µL. The combi pal wash 1 (95% water/5% acetonitrile) and wash 2 (5% water/95% acetonitrile) was used to minimize carryover.

**Table 4:** Retention times (RT) and MS parameters: collision cell entrance potential (CEP) for precursor ions and collision energy (CE), collision cell exit potential (CXP) and the resulting ion ratios for the product ions.

| Analyte | $t_R$ (min) | Precursor Ions | Product Ions | Median ion ratio, quant/ qual % | DP | CE | CXP |
|---|---|---|---|---|---|---|---|
| emamectin | 2.83 | 886.8 | **158** | 100 | 66 | 39 | 36 |
| | | | 126.2 | 50.9 | | 47 | 16 |
| | | | 302 | 16 | | 30 | 16 |
| eprinomectin | 3.42 | 914.2 | **186.1** | 100 | 71 | 25 | 30 |
| | | | 154.1 | 44 | | 49 | 14 |
| | | | 298.2 | 40 | | 27 | 18 |
| abamectin* | 3.98 | 890.5 | **567.2** | 100 | 61 | 21 | 16 |
| | | | 305.4 | 98 | | 31 | 38 |
| | | | 145.2 | 20 | | 47 | 20 |

| Analyte | $t_R$ (min) | Precursor Ions | Product Ions | Median ion ratio, quant/ qual % | DP | CE | CXP |
|---|---|---|---|---|---|---|---|
| doramectin* | 4.40 | 916.6 | **331.1** | 100 | 66 | 35 | 44 |
| | | | 593.4 | 72 | | 21 | 7 |
| | | | 145.1 | 16 | | 39 | 16 |
| moxidectin | 4.89 | 640.3 | **528.3** | 100 | 41 | 14 | 15 |
| | | | 498.3 | 63 | | 17 | 14 |
| | | | 199.1 | 24 | | 27 | 24 |
| selamectin | 4.98 | 770.4 | 608.3 | 100 | 36 | 29 | 20 |
| | | | 145.2 | 23 | | 41 | 10 |
| | | | 105.1 | 15 | | 109 | 20 |
| ivermectin* | 5.01 | 892.6 | **307.2** | 100 | 76 | 35 | 14 |
| | | | 551.4 | 55 | | 31 | 16 |
| | | | 145.1 | 15 | | 57 | 16 |

*-The precursor ion is the ammonium adduct

## RESULTS AND DISCUSSION

### *Method Validation*

The objective of this research was to validate a sensitive and rapid method for the determination of macrocyclic lactones in game meat tissue. The method was validated according to the FDA OFVM Level Two Chemical Method Validation guidelines[7]. The analyte concentration levels tested in the method validation were similar to the levels published in LIB 4496[2]. Thus, the 1VL validation level was 10 ng/g for all compounds, and the calibration levels ranged from 2.50 ng/g to 80.0 ng/g. For routine regulatory analysis, the calibration range can be decreased to 5 ng/g- 80 ng/g.

Three validation levels were tested corresponding to concentrations of 0.5VL, 1VL, and 2VL corresponding to 5 ng/g, 10 ng/g, and 20 ng/g. Validation studies were carried out on muscle tissue (bison, deer, elk, and rabbit) and liver tissue (bison, bovine). Negative controls were verified to be free of macrocyclic lactone prior to validation studies. A total of 84 calibration curves were analyzed during the validation of all seven analytes and matrices. Each matrix was tested over a two-day period. The correlation coefficient ($r^2$) was averaged over 2 days per matrix and ranged from 0.9950 – 0.9999 for all matrices.

Accuracy and precision results from the validation are summarized in Table 5 for all six matrices. Method accuracy (trueness) was determined by calculating the recoveries of analytes in each matrix calibration curve based on extracted matrix calibrants. The results demonstrated the method accuracy was satisfactory for all compounds and matrices, according to FDA OFVM guidance[7]. Average recoveries for each analyte in each matrix ranged from 78.3 to 113 %, as shown in Table 5. Analyte recoveries averaged among all six matrices ranged from 93.7 to 97.1%, within the range of 60%-115% specified for quantitative residue analysis at the concentration range of 5 to 20 ng/g (ppb)[7].

Method detection levels (MDLs) were calculated from the quantitative product ion transition for each analyte in each matrix and are reported in Table 5. The method detection limit was evaluated by analyzing ten replicates fortified at the 0.5VL concentration, where MDL = t*s (“t” is the Student's t values at the 99% confidence level, and “s” is the standard deviation of the tested concentration. The lower limit of quantification (LOQ) was calculated by taking the sd of the 0.5VL level by a factor of 10, refer to Table 5.

**Table 5: Accuracy and precision combined from all seven matrices**

| Fortification Level | Trueness (as % Recovery) ± %RSD | | | | | | |
|---|---|---|---|---|---|---|---|
| (µg/kg) | ABA | DOR | EMA | EPR | MOX | IVR | SEL |
| **Bovine Liver** | | | | | | | |
| 5.00 | 88.2 ± 2.47 | 89.5 ± 3.40 | 83.5 ± 7.38 | 85.0 ± 2.73 | 86.1 ± 3.16 | 80.4 ± 4.32 | 83.7 ± 6.46 |
| 10.0 | 96.0 ± 4.86 | 96.3 ± 5.54 | 97.2 ± 8.77 | 98.2 ± 4.25 | 92.2 ± 2.87 | 97.6 ± 3.39 | 96.3 ± 3.25 |
| 20.0 | 101 ± 2.80 | 103 ± 1.43 | 106 ± 2.82 | 102 ± 5.93 | 100 ± 1.82 | 102 ± 5.26 | 108 ± 6.85 |
| MDL (µg/kg) | 1.09 | 0.57 | 1.16 | 0.44 | 0.51 | 0.65 | 1.01 |
| LOQ (µg/kg) | 0.408 | 1.52 | 3.10 | 1.16 | 1.34 | 1.74 | 2.70 |
| **Bison Liver** | | | | | | | |
| 5.00 | 90.1 ± 5.25 | 97.0 ± 2.51 | 95.1 ± 6.96 | 91.1 ± 10.5 | 94.0 ± 10.5 | 96.9 ± 6.66 | 80.6 ± 15.3 |
| 10.0 | 85.7 ± 7.13 | 92.0 ± 6.64 | 94.5 ± 3.23 | 90.3 ± 3.21 | 90.1 ± 8.10 | 92.9± 9.23 | 79.7 ± 4.31 |
| 20.0 | 90.7 ± 3.46 | 97.1 ± 3.87 | 94.3 ± 3.34 | 94.7 ± 2.88 | 90.4 ± 5.25 | 94.6 ±3.68 | 78.3 ± 4.19 |
| MDL (µg/kg) | 0.67 | 0.91 | 3.31 | 1.35 | 1.39 | 0.91 | 1.73 |
| LOQ (µg/kg) | 2.37 | 3.22 | 0.930 | 4.80 | 4.99 | 3.22 | 6.15 |
| **Bison Muscle** | | | | | | | |
| 5.00 | 98.6 ± 4.15 | 103 ± 1.50 | 84.5 ± 12.1 | 92.0 ± 7.47 | 86.6 ±9.30 | 98.8 ± 7.20 | 91.6 ± 7.50 |
| 10.0 | 105 ± 9.70 | 98.3 ± 3.30 | 99.7 ± 10.6 | 93.9 ± 8.68 | 89.4 ± 12.3 | 99.5 ± 11.6 | 94.7 ± 10.3 |
| 20.0 | 95.9 ± 5.77 | 104 ± 1.50 | 89.5 ± 9.96 | 95.8 ± 6.38 | 77.2 ± 13.2 | 95.2 ± 12.3 | 84.4 ± 14.0 |
| MDL (µg/kg) | 0.58 | 1.00 | 5.12 | 0.97 | 1.14 | 1.08 | 0.97 |
| LOQ (µg/kg) | 2.05 | 3.56 | 1.44 | 3.43 | 4.03 | 3.86 | 3.43 |
| **Deer (venison) Muscle** | | | | | | | |
| 5.00 | 97.4 ± 6.76 | 90.5 ± 12.9 | 96.1 ± 6.57 | 99.1 ± 3.15 | 90.9 ± 9.02 | 97.7 ± 3.10 | 102 ± 5.18 |
| 10.0 | 95.1 ± 6.47 | 88.7 ± 7.28 | 94.5 ± 6.65 | 93.2 ± 8.92 | 87.2 ± 4.36 | 90.8 ± 7.03 | 99.7 ± 3.53 |
| 20.0 | 94.2 ± 3.98 | 94.4 ± 2.96 | 93.9 ± 3.01 | 93.4 ± 3.25 | 89.9 ± 3.43 | 93.4 ± 3.25 | 99.3 ± 4.12 |
| MDL (µg/kg) | 4.75 | 1.65 | 0.89 | 0.446 | 1.38 | 1.53 | 0.74 |
| LOQ (µg/kg) | 1.34 | 5.84 | 3.12 | 1.56 | 3.77 | 2.93 | 2.63 |
| **Elk Muscle** | | | | | | | |
| 5.00 | 85.6 ± 3.11 | 89.1 ± 3.69 | 79.1 ± 4.57 | 79.4 ± 3.68 | 87.0 ± 7.14 | 85.5 ± 3.66 | 89.0 ± 4.45 |
| 10.0 | 103 ± 4.72 | 106 ± 3.78 | 93.9 ± 10.4 | 106 ± 6.62 | 109 ± 3.33 | 113 ± 4.10 | 104 ± 4.33 |
| 20.0 | 101 ± 6.59 | 98.5 ± 5.14 | 89.1 ± 6.24 | 103 ± 8.11 | 99.3 ± 3.28 | 110 ± 5.57 | 107 ± 4.92 |
| MDL (µg/kg) | 1.33 | 0.464 | 0.51 | 0.41 | 0.88 | 0.44 | 0.60 |
| LOQ (µg/kg) | 0.38 | 1.65 | 1.81 | 1.46 | 3.11 | 1.57 | 1.98 |
| **Rabbit Muscle** | | | | | | | |
| 5.00 | 91.0 ± 4.76 | 92.4 ± 9.16 | 97.3 ± 6.23 | 94.1 ± 9.98 | 96.3 ± 6.79 | 90.1 ± 8.56 | 96.5 ± 5.92 |
| 10.0 | 100 ± 84.2 | 96.9 ± 4.83 | 100.1 ± 2.5 | 103 ± 6.35 | 101 ± 9.59 | 99.1 ± 10.2 | 92.5 ± 2.73 |
| 20.0 | 101 ± 4.31 | 110 ± 4.22 | 97.1 ± 4.0 | 103 ± 4.95 | 113 ± 2.98 | 110 ± 2.30 | 101 ± 9.73 |
| MDL (µg/kg) | 0.61 | 1.19 | 0.86 | 1.33 | 0.92 | 1.09 | 0.81 |
| LOQ (µg/kg) | 2.17 | 4.23 | 3.03 | 4.70 | 3.27 | 3.86 | 2.86 |

### *Quantitative Analysis*

For routine regulatory sample analysis, it is labor intensive to analyze a set of five matrix-matched extracted calibrants with every analysis. The burden increases if more than one type of matrix is present in the set of regulatory samples (e.g., bison and rabbit) and multiple sets of matrix-matched calibrants must be extracted and analyzed to perform the analysis. Due to the different matrices involved each type of matrix required a separate matrix-matched extracted calibration curve.

***Qualitative Identification***

The FDA CVM 118 guidance[9] provides identification criteria to determine if a residue can be identified. It states that the LC-MS analysis yields a chromatographic peak within ± 5% retention time of the chromatographic peak relative to the standard, and the chromatographic peak should exceed a signal-to-noise (s/n) threshold of 3:1. In addition, two qualitative product ion ratios must be within ± 20% (absolute) of the ion ratios from the average of the five extracted calibrant standards analyzed in the same sequence.

***Regulatory Sample Analysis***

Approximately 100 bison liver regulatory samples have been analyzed by this method. Two bison samples, were found positive for ivermectin (6.97 ng/g) or eprinomectin (9.32 ng/g). The sample positive for ivermectin was below the 15 ng/g regulatory level. The samples provided by USDA FSIS also included the muscle tissue from the animal, hence the muscle samples were also analyzed for avermectins. Neither ivermectin or eprinomectin were detected in the muscle samples above the reported MLDs, Table 5. Figures 1-2 give an example of the extracted ion chromatogram for negative control bison liver and an extracted calibrant standard-2 at 10 ng/g; Figure 3-5 are the Multi Quant reports showing typical extracted ion chromatograms of bison liver matrix negative control, extracted std-3, positive ivermectin samples. Similar reports for eprinomectin are shown in Figures 6-8. The figures also demonstrate the ion ratios based on the average extracted calibration curve.

***Investigation of Matrix Effects and Solvent Calibrant Performance***

This method was based on LIB 4496 (FY11)[2] and Hernando et al.[10]. Both publications report on investigation of solvent curves, matrix matched and extracted curves, and matrix effect. The extent of matrix effects (ME) and efficiency of the extraction process (RE) were calculated based on the method by Gosetti et al.[11]. Solvent calibrants (A), post-fortified matrix calibrants (B), and extracted matrix calibrants (C) were prepared for the bison liver matrix and analyzed to determine ME and RE using the equation:

ME (%) = B/A*100
RE (%) = C/B*100

As was demonstrated with the previous publications and the experiments for this LIB, there was signification matrix effect for IVR, DOR, MOX, and SEL. The average ME for these analysts was approximately 35% and the average RE was 65%. The use of solvent curves yielded unacceptable recoveries for IVR, DOR, MOX, and SEL not only for bison liver, but all matrices had below 40% at all validation levels. These results support the use of extracted matrix calibrants for macrocyclic lactone residue analysis in bison, deer, elk, and rabbit tissues.

**CONCLUSION**

A method was validated for the determination of seven macrocyclic lactone compounds in muscle and liver tissues from different animal species (bison, venison, elk, and rabbit). Since the initial start of this program in 2014, approximately 100 bison liver samples have been analyzed for MLs with two samples determined to be positive for ivermectin and eprinomectin at concentrations of 6.97ng/g and 9.32 ng/g, respectively. The corresponding muscle tissues were analyzed and no avermectins were determined above the reported MDLs.

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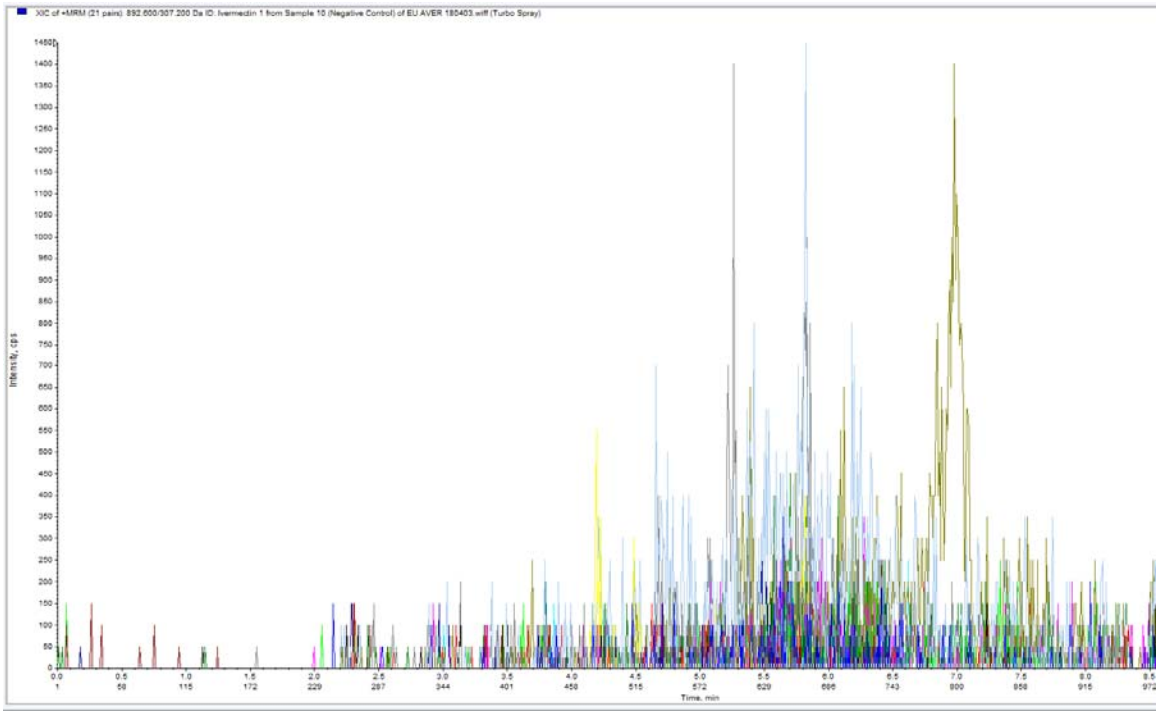

**Figure 1: XIC of +MRM (21 pairs); Bison Negative Control**

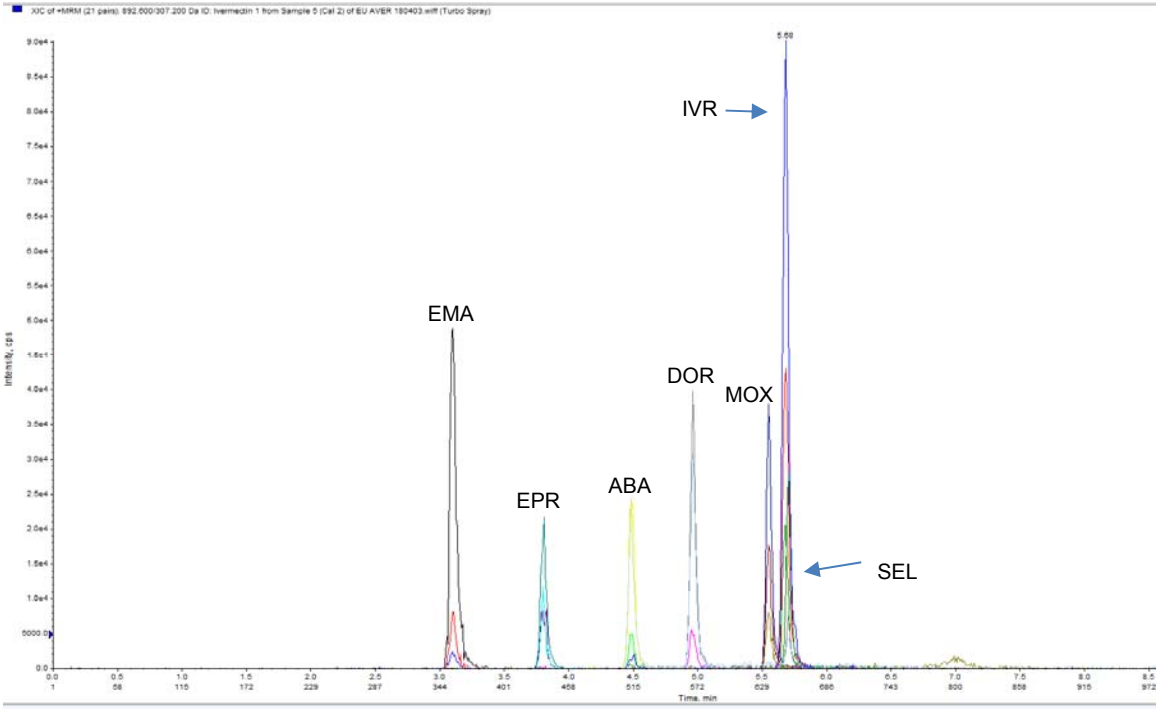


**Figure 2: XIC of +MRM (21 pairs); Extracted Std-2 (10 ng/g) in Negative Control Bison**

| | | | |
|---|---|---|---|
| Project | Avermectins\2015_05_21 | Data File | EU AVER 170424.wiff |
| Result Table | EU AVER 170324 Extracted Curve IVR | Result Table Date | 3/27/2017 11:15:03 AM |
| Sample Name | Negative Control (Bison) | Injection Volume | 10.00 |
| Injection Date | 3/24/2017 11:34:11 AM | Injection Vial | 8 |
| Operator | ORI4WDEN1701756\FDA3 | Sample Type | Quality Control |
| Acquisition Method | EU AVER 170422.dam | Dilution Factor | 1.00 |
| Instrument Name | QTRAP 5500 | | |

**Results Summary**

| Analyte Peak Name | Analyte RT | Expected RT | Calc Conc. (ng/g) | Analyte Response | Calculated Ion Ratio (Expected Value) | Ratio Confirms |
|---|---|---|---|---|---|---|
| Ivermectin 1 | N/A | 5.48 | N/A | N/A | | |
| Ivermectin 2 | N/A | 5.47 | | N/A | 0.0% (48.1%) | |
| Ivermectin 3 | **N/A** | **5.47** | | N/A | **0.0% (17.2%)** | ✔ |

**Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions**

**Ivermectin 1**

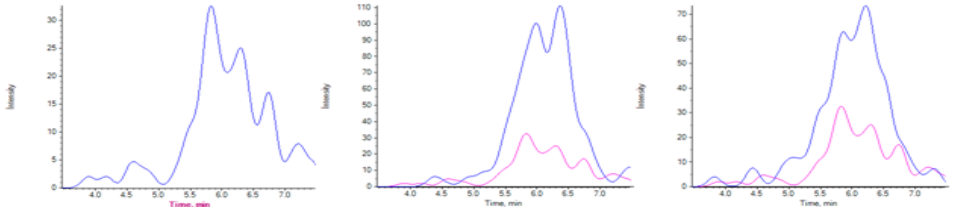

**Figure 3: Ivermectin Multi Quant Report Negative Control Bison Liver:**
Ivermectin 1 Quantitation ion (m/z 892.9 → 307.2),
Ivermectin 2 Confirmation (m/z 892.9 → 551.4) & Ivermectin 3 (m/z 892.9 →145.1)

| | | | |
|---|---|---|---|
| Project | Avermectins\2015_05_21 | Data File | EU AVER 170424.wiff |
| Result Table | EU AVER 170324 Extracted Curve IVR | Result Table Date | 3/27/2017 11:15:03 AM |
| Sample Name | Extracted Std-3 | Injection Volume | 10.00 |
| Injection Date | 3/24/2017 10:47:11 AM | Injection Vial | 5 |
| Operator | ORI4WDEN1701756\FDA3 | Sample Type | Standard |
| Acquisition Method | EU AVER 170422.dam | Dilution Factor | 1.00 |
| Instrument Name | QTRAP 5500 | | |

**Results Summary**

| Analyte Peak Name | Analyte RT | Expected RT | Calc Conc. (ng/g) | Analyte Response | Calculated Ion Ratio (Expected Value) | Ratio Confirms |
|---|---|---|---|---|---|---|
| Ivermectin 1 | 5.47 | 5.48 | 19.95 | 422040.0 | | |
| Ivermectin 2 | **5.47** | **5.47** | | 200834 | **47.6% (48.1%)** | ✔ |
| Ivermectin 3 | **5.47** | **5.47** | | 72694 | **17.2% (17.2%)** | ✔ |

**Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions**

**Ivermectin 1**

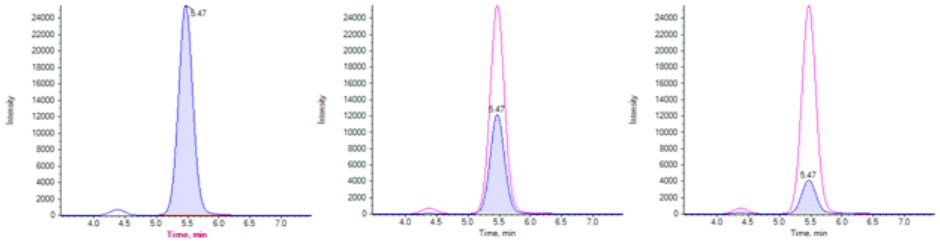

**Figure 4: Ivermectin Multi Quant Report Extracted Std-3 20 ng/g Bison Liver:**
Ivermectin 1 Quantitation ion (m/z 892.9 → 307.2),
Ivermectin 2 Confirmation (m/z 892.9 → 551.4) & Ivermectin 3 (m/z 892.9 →145.1)

| | | | |
|---|---|---|---|
| Project | Avermectins\2015_05_21 | Data File | EU AVER 170424.wiff |
| Result Table | EU AVER 170324 Extracted Curve IVR | Result Table Date | 3/27/2017 11:15:03 AM |
| Sample Name | 1003924 -Bison Liver | Injection Volume | 10.00 |
| Injection Date | 3/24/2017 12:52:33 PM | Injection Vial | 13 |
| Operator | ORI4WDEN1701756\FDA3 | Sample Type | Unknown |
| Acquisition Method | EU AVER 170422.dam | Dilution Factor | 1.00 |
| Instrument Name | QTRAP 5500 | | |

**Results Summary**

| Analyte Peak Name | Analyte RT | Expected RT | Calc Conc. (ng/g) | Analyte Response | Calculated Ion Ratio (Expected Value) | Ratio Confirms |
|---|---|---|---|---|---|---|
| Ivermectin 1 | 5.47 | 5.48 | 6.79 | 154932.0 | | |
| Ivermectin 2 | **5.47** | **5.47** | | 76986 | **49.7% (48.1%)** | ✔ |
| Ivermectin 3 | **5.47** | **5.47** | | 28455 | **18.4% (17.2%)** | ✔ |

**Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions**

**Ivermectin 1**

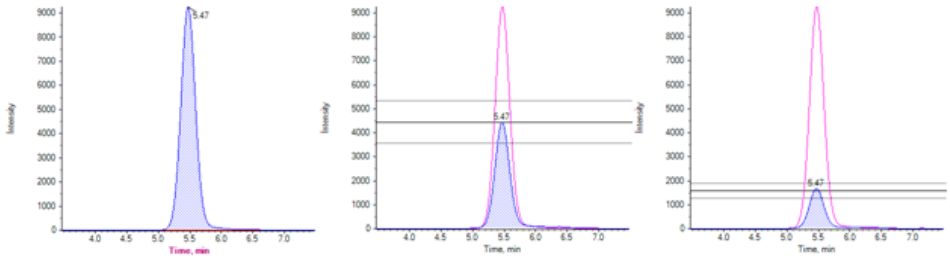


**Figure 5: Ivermectin Multi Quant Report Postive Sample in Bison Liver:**
Ivermectin 1 Quantitation ion (m/z 892.9 → 307.2),
Ivermectin 2 Confirmation (m/z 892.9 → 551.4) & Ivermectin 3 (m/z 892.9 →145.1)

| | | | |
|---|---|---|---|
| Project | Avermectins in Game Meat\2015_03_11 | Data File | EU AVER 171127.wiff |
| Result Table | EU AVER EPR conf 171127 | Result Table Date | 11/28/2017 8:15:39 AM |
| Sample Name | Negative Control | Injection Volume | 10.00 |
| Injection Date | 11/27/2017 3:57:39 PM | Injection Vial | 10 |
| Operator | ORI4WDEN1701726\fda | Sample Type | Quality Control |
| Acquisition Method | EU Aver EPR only.dam | Dilution Factor | 1.00 |
| Instrument Name | QTRAP 5500 | | |

**Results Summary**

| Analyte Peak Name | Analyte RT | Expected RT | Calc Conc. (ng/g) | Analyte Response | Calculated Ion Ratio (Expected Value) | Ratio Confirms |
|---|---|---|---|---|---|---|
| Eprinomectin 1 | N/A | 3.93 | N/A | N/A | | |
| Eprinomectin 2 | 4.05 | 3.82 | | 87 | 0.0% (42.7%) | |
| Eprinomectin 3 | 3.89 | 3.83 | | 583 | 0.0% (40.6%) | |

**Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions**

Eprinomectin 1

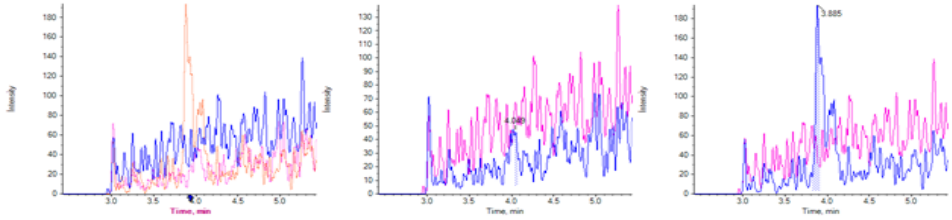

**Figure 6: Eprinomectin Multi Quant Report Negative Control Bison Liver:**
Eprinomectin 1 Quantitation ion (m/z 914.2 → 186.1),
Eprinomectin 2 Confirmation (m/z 914.2 → 154.1) & Eprinomectin 3 (m/z 914.2 →298.1)

| | | | |
|---|---|---|---|
| Project | Avermectins in Game Meat\2015_03_11 | Data File | EU AVER 171127.wiff |
| Result Table | EU AVER EPR conf 171127 | Result Table Date | 11/28/2017 8:15:39 AM |
| Sample Name | Ext Std 3 - 20 ng/g | Injection Volume | 10.00 |
| Injection Date | 11/27/2017 2:53:35 PM | Injection Vial | 5 |
| Operator | ORI4WDEN1701726\fda | Sample Type | Standard |
| Acquisition Method | EU Aver EPR only.dam | Dilution Factor | 1.00 |
| Instrument Name | QTRAP 5500 | | |

**Results Summary**

| Analyte Peak Name | Analyte RT | Expected RT | Calc Conc. (ng/g) | Analyte Response | Calculated Ion Ratio (Expected Value) | Ratio Confirms |
|---|---|---|---|---|---|---|
| Eprinomectin 1 | 3.86 | 3.93 | 19.34 | 173158.0 | | |
| Eprinomectin 2 | **3.86** | **3.82** | | 71768 | **41.4% (42.7%)** | ✔ |
| Eprinomectin 3 | **3.86** | **3.83** | | 68860 | **39.8% (40.6%)** | ✔ |

**Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions**

Eprinomectin 1

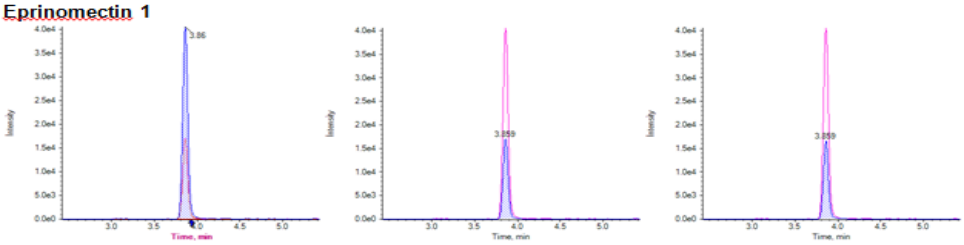

**Figure 7: Eprinomectin Multi Quant Report Extracted Std-3 20 ng/g Bison Liver:**
Eprinomectin 1 Quantitation ion (m/z 914.2 → 186.1),
Eprinomectin 2 Confirmation (m/z 914.2 → 154.1) & Eprinomectin 3 (m/z 914.2 →298.1)

| Project | Avermectins in Game Meat\2015_03_11 | Data File | EU AVER 171127.wiff |
|---|---|---|---|
| Result Table | EU AVER EPR conf 171127 | Result Table Date | 11/28/2017 8:15:39 AM |
| Sample Name | 1030444 bison liver | Injection Volume | 10.00 |
| Injection Date | 11/27/2017 4:29:41 PM | Injection Vial | 13 |
| Operator | ORI4WDEN1701726\fda | Sample Type | Unknown |
| Acquisition Method | EU Aver EPR only.dam | Dilution Factor | 1.00 |
| Instrument Name | QTRAP 5500 | | |

**Results Summary**

| Analyte Peak Name | Analyte RT | Expected RT | Calc Conc. (ng/g) | Analyte Response | Calculated Ion Ratio (Expected Value) | Ratio Confirms |
|---|---|---|---|---|---|---|
| Eprinomectin 1 | 3.83 | 3.93 | 9.32 | 82185.0 | | |
| Eprinomectin 2 | **3.83** | **3.82** | | 36294 | **44.2% (42.7%)** | ✓ |
| Eprinomectin 3 | **3.83** | **3.83** | | 34471 | **41.9% (40.6%)** | ✓ |

**Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions**

**Eprinomectin 1**

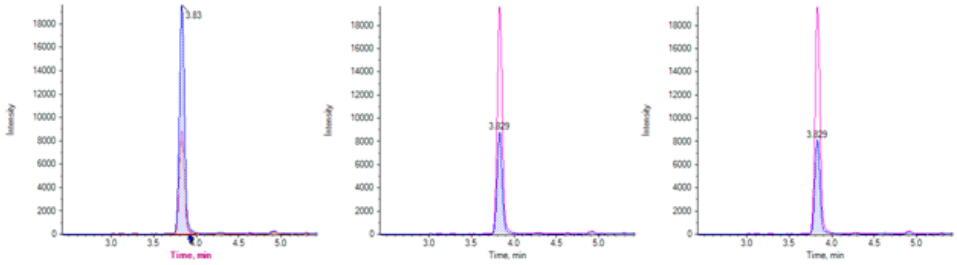


**Figure 8: Eprinomectin Multi Quant Report Postive Bison Liver:**
Eprinomectin 1 Quantitation ion (m/z 914.2 → 186.1),
Eprinomectin 2 Confirmation (m/z 914.2 → 154.1) & Eprinomectin 3 (m/z 914.2 →298.1)